

FINAL REPORT

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**NUCLEATION AND CONVECTION EFFECTS
IN PROTEIN CRYSTAL GROWTH**

Period of performance

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Our work under this grant has significantly contributed to the goals of the NASA supported protein crystallization program. We have achieved the main objectives of the proposed work, as outlined in the original proposal:

- I. We have provided important insight into protein nucleation and crystal growth mechanisms to facilitate a rational approach to protein crystallization.
- II. We have delineated the factors that currently limit the x-ray diffraction resolution of protein crystals, and their correlation to crystallization conditions.
- III. We have developed novel technologies to study and monitor protein crystal nucleation and growth processes, in order to increase the reproducibility and yield of protein crystallization.

We have published 17 papers in peer-reviewed scientific journals and books and made more than 15 invited and 9 contributed presentations of our results at international and national scientific meetings. A list of these publications is presented at the end of this report, a copy of each paper is attached to the report.

Towards our **first** objective, we have studied the interaction between protein molecules in the solution that underlie their ability to crystallize, liquid-liquid phase separations that may accompany and enhance or prevent nucleation of protein crystals, and we have continued our detailed studies of defect-causing instabilities in the crystal growth kinetics that provide an insight into the effects of microgravity on protein crystal perfection.

Liquid-liquid phase separations in supersaturated lysozyme solutions and associated precipitate formation. Using cloud point determinations, the phase boundaries (binodals) for metastable liquid-liquid (L-L) separation in supersaturated hen egg white lysozyme solutions with 3 %, 5 %, and 7 % (w/v) NaCl at pH = 4.5 and protein concentrations c between 40 and 400 mg/ml were determined. The critical temperature for the binodal increased approximately linearly with salt concentration. The coexisting liquid phases both remained supersaturated but differed widely in protein concentration. No salt repartitioning was observed between the initial solution. At high initial solution. At high initial protein concentrations, a metastable gel phase formed at temperatures above the liquid binodal. Both crystal nucleation and gel formation were accelerated in samples that had been cycled through the binodal. Solutions in the gel and L-L regions yielded various types of precipitates. Based on theoretical considerations, previous observations with other proteins, and our experimental results with lysozyme, a generic phase diagram for globular proteins is put forth. A limited region in the (T, c) plane favorable for the growth of protein single crystals is delineated.

Interactions and lack of pre-nucleation aggregates in lysozyme solutions. There have been numerous claims of large concentrations of prenucleation aggregates in supersaturated as well as undersaturated lysozyme solutions at high salt concentrations. The presence of these aggregates was derived from

measurements of the light or neutron scattering intensity, ultracentrifugation and dialysis behavior, as well as over-simplified crystal growth kinetics considerations. In all these interpretations it has been assumed that lysozyme solutions are either ideal or that protein interactions are independent of salt concentration. Contrary to these presumptions, our static and dynamic light scattering experiments provide evidence that lysozyme forms highly non-ideal, strongly interacting solutions. At low salt concentrations, the scattering intensities fall well below the values expected for an ideal, monomeric solution at the same protein concentration, while diffusivities increase with increasing protein concentration. Upon increase in salt concentration, these trends are eventually reversed. This enhancement in scattering intensity and decrease in diffusivity was widely interpreted as sign of aggregate formation. Yet, a quantitative interpretation of the scattering behavior over the whole salt concentration range can only be given in terms of a transition from net repulsion to net attraction between lysozyme monomers. Increased salt screening of the electrostatic repulsion among the protein macro-ions, together with attractive protein interactions, such as van der Waals, hydrophobic and hydration forces, provide an unambiguous mechanism for the observed transition and a more physical interpretation of the various observations.

Nucleation and crystallization of globular proteins - what do we know and what is missing? Recently, much progress has been made in understanding the nucleation and crystallization of globular proteins, including the formation of compositional and structural crystal defects. Insight into the interactions of (screened) protein macro-ions in solution, obtained from light scattering studies, can guide the search for crystallization conditions. These studies show that the nucleation of globular proteins is governed by the same principles as that of small molecules. However, failure to account for direct and indirect (hydrodynamic) protein interactions in the solutions results in unrealistic aggregation scenarios. Microscopic studies, mostly performed with hen-egg-white lysozyme (HEWL), reveal that crystals grow by the attachment of growth units through the same layer-spreading mechanisms as inorganic crystals. Growth kinetics are found to be nonsteady under steady external conditions. Long-term variations in growth rates are due to changes in step-originating dislocation groups. Fluctuations on a shorter time-scale appear to result from some nonlinear interplay between interfacial kinetics and bulk transport. Systematic gel electrophoretic analyses suggest that most HEWL crystallization studies have been performed with material containing other proteins at percent levels. Yet, sub-percent levels of protein impurities have been shown to impede growth step propagation and to play a role in the formation of structural/compositional inhomogeneities. In crystal growth from highly purified HEWL solutions, however, such inhomogeneities are much weaker and form only in response to unusually large changes in growth conditions. Equally important for connecting growth conditions to crystal perfection and diffraction resolution are recent advances in structural characterization through high resolution Bragg reflection profiling and X-ray topography.

A rationale for system-dependent advantages and disadvantages of solution crystal growth at low gravity. Protein crystallization experiments at reduced gravity have yielded crystals that, depending on the specific

material, are either superior or inferior in their structural perfection compared to counterparts grown at normal gravity. A reduction of the crystals' quality due to their growth at low gravity cannot be understood from existing models. Our experimental investigations of the ground-based crystallization of the protein lysozyme have revealed pronounced unsteady growth layer dynamics and associated defect formation under steady external conditions. Through scaling analysis and numerical simulations we show that the observed fluctuations originate from the coupling of bulk transport with nonlinear interface kinetics under mixed kinetics-transport control of the growth rate. The amplitude of the fluctuations is smallest when either transport or interfacial kinetics dominate the control of the crystallization process. Thus, depending on the specific system, crystal quality may be improved by either enhancing or suppressing the transport in the solution. These considerations provide, for the first time, a material-dependent rationale for the advantages, as well as disadvantages, of reduced gravity for (protein) crystallization.

Towards our **second** main objective, we have studied *type II striations* that form in crystals even under steady external conditions, impurity and salt rich *cores* that form in the centers of growing crystals, *impurity* adsorption on the interface, effects on the surface structure and crystal growth kinetics consequences of impurity incorporation into the crystals, defects and lattice strain by *x-ray topography*.

Intrinsic kinetics fluctuations as cause of growth inhomogeneity in protein crystals. Intrinsic kinetics instabilities in the form of growth step bunching during the crystallization of the protein lysozyme from solution were characterized by *in situ* high-resolution optical interferometry. Compositional variations (striations) in the crystal, that potentially decrease its utility, e.g., for molecular structure studies by diffraction methods, were visualized by polarized light reflection microscopy. Spatio-temporal correlation was established between the sequence of moving step bunches and the striations

Repartitioning of NaCl and protein impurities in lysozyme crystallization Nonuniform precipitant and impurity incorporation in protein crystals can cause lattice strain and, thus, possibly decrease the X-ray diffraction resolution. To address this issue, a series of crystallization experiments were carried out, in which initial supersaturation, NaCl concentration, protein purity level and crystallized fraction were varied. Lysozyme and protein impurities, as well as sodium and chloride were independently determined in the initial solution, supernatant and crystals. The segregation coefficients for Na^+ and Cl^- were found to be independent of supersaturation and NaCl concentration, and decreased with crystallized fraction/crystal size. Numerical evaluation of the extensive body of data, based on a nucleation-growth-repartitioning model, suggests a core of $\sim 40 \mu\text{m}$ in which salt is incorporated in much greater concentrations than during later growth. Small crystals containing higher amounts of incorporated NaCl also had higher protein impurity contents. This suggests that the excess salt is associated with the protein impurities in the core. X-ray topography revealed strain fields in the crystals' centers comparable in size to the inferred core. The growth

rates of crystals smaller than 30-40 μm size were consistently 1.5-2 times lower than those of larger crystals, presumably due to higher chemical potentials in the core.

Effects of microheterogeneity on hen egg white lysozyme crystallization. In earlier SDS PAGE studies we found that commonly utilized commercial hen egg white lysozyme (HEWL) preparations contained 0.2–0.4 mol% covalently bound dimers. Here we show, using high performance capillary electrophoresis (HPCE), that HEWL contains, in addition, two differently charged monomers in comparable amounts. To explore the origin of these microheterogeneous contaminants, we have oxidized purified HEWL (PHEWL) with hydrogen peroxide (0.0026–0.88 M) at various pH between 4.5–12.0. Optical densitometry of oxidized PHEWL (OHEWL) bands in SDS PAGE gels shows that hydrogen peroxide at 0.88 M in acetate buffer pH 4.5 increased the amount of dimers about six-fold over that in commercial HEWL. OHEWL had, in addition to one of the two monomer forms found in HEWL and PHEWL, three other, differently charged monomer forms, each of them representing about 25% of the preparation. SDS PAGE analysis of OHEWL yielded two closely spaced dimer bands with $M_r = 28,000$ and $27,500$. In addition, larger HEWL oligomers with $M_r = 1.7$ million and $320,000$ were detected by Gel Filtration Fast Protein Liquid Chromatography with multiangle laser light scattering detection. Nondissociating PAGE in large pore size gels at pH 4.5 confirmed the presence of these large oligomers in HEWL and OHEWL. Increased microheterogeneity resulted in substantial effects on crystal growth and nucleation rate. On addition of $10\ \mu\text{g}$ – $1\ \text{mg/ml}$ OHEWL to $32\ \text{mg/ml}$ HEWL crystallizing solutions, both the number and size of forming crystals decreased roughly proportional to the concentration of the added microheterogeneity. The same effect was observed in HEWL solutions on addition of 0.03–0.3 M hydrogen peroxide. Repartitioning of the dimer during crystallization at various temperatures between 4 and $20\ ^\circ\text{C}$ was analyzed by SDS PAGE. The crystals contained = 25% (w/w) of the oligomers in the solution, with no apparent temperature dependence of the repartitioning.

Effect of microheterogeneity on horse spleen apoferritin crystallization. Apoferritin (APO) is an interesting model protein for *crystal* growth studies, as an alternative to the widely used hen egg white lysozyme. The effect of naturally occurring oligomers on the crystallization of isolated, microhomogeneous APO monomers (24 subunits, $M_r = 440,000$) was investigated. SDS PAGE analysis and immunoblotting showed that commercial APO was free of foreign proteins (>99.9% w/w). The quaternary structure of APO oligomers that form prior to the addition of precipitant was analyzed in native 4–15% T (1–2% C) gradient PAGE. Optical densitometry of these gels showed that oligomers (> 24 subunit monomer) constituted approximately 45% w/w of the total APO. The primary oligomeric contaminants were dimers (48 subunits) with 35% w/w, and several bands constituting trimers (~72 subunits) with 10% w/w. Directly determined physical molecular weights (M_w) and conformational data for oligomers obtained by analytical gel filtration Fast Protein Liquid Chromatography separations utilizing UV and multi-angle laser light scattering detectors (GF-FPLC-MALLS) confirmed and expanded the native PAGE results. This technique allowed

the discovery of large oligomers ($M_w = 5,000,000$ and $80,000,000$) present in concentrations $< 1\%$ w/w. Semi-preparative GF-FPLC was used to quantitatively reduce oligomer contamination to 5% w/w, and to produce 0.25 g of microhomogeneous monomers from 0.5 g APO. Crystallization from microhomogeneous monomer solutions yielded large crystals 0.5 - 1.0 mm in size. These crystals yielded an X-ray diffraction resolution of 1.8 Å. Reconstitutive experiments in which isolated oligomers were added to monomer preparations showed that dimers perturb the growth habit and reduce the crystal growth, without significantly affecting the nucleation. On trimer addition, the nucleation was increased and the crystal growth decreased. Addition of cadmium sulfate precipitant to unpurified APO did not affect the nature or quantity of the oligomers. These effects of oligomers on crystallization underline microheterogeneity as a critical factor in protein crystallization.

Effects of convective solute and impurity transport in protein crystal growth. High-resolution optical interferometry was used to investigate the effects of forced solution convection on the crystal growth kinetics of the model protein lysozyme. Most experiments were conducted with 99.99% pure protein solutions. To study impurity effects, $\sim 1\%$ of lysozyme dimer (covalently bound) was added in some cases. We show that the unsteady kinetics, corresponding to bunching of growth steps, can be characterized by the Fourier components of time-traces of the growth rate. Specific Fourier spectra are uniquely determined by the solution conditions (composition, temperature and flow rate) and the growth layer source activity. We found that the average step velocity and growth rate increase by $\sim 10\%$ with increasing flow rate, as a result of the enhanced solute supply to the interface. More importantly, faster convective transport results in lower fluctuation amplitudes. This observation supports a rationale for system-dependent effects of transport on the structural perfection of protein crystals [Vekilov et al., *Phys. Rev.* **1996**, *E54*, 6650]. We also found that solution flow rates > 500 $\mu\text{m/s}$ result in stronger fluctuations while the average growth rate is decreased. This can lead to growth cessation at low supersaturations. With the intentionally contaminated solutions, these undesirable phenomena occurred at about half the flow rates required in pure solutions. Thus, we conclude that they are due to enhanced convective supply of impurities which are incorporated into the crystal during growth. Furthermore, we found that the impurity effects are reduced at higher crystal growth rates. Since the exposure time of terraces is inversely proportional to the growth rate, this observation suggests that the increased kinetics instability results from impurity adsorption on the interface. Finally, we provide evidence relating earlier observations of "slow protein crystal growth kinetics" to step bunch formation in response to nonsteady step generation.

X-ray topography of tetragonal Lysozyme grown by the temperature controlled technique Growth-induced defects in lysozyme crystals were studied by white beam and monochromatic X-ray topography at the National Synchrotron Light Source (NSLS) at the Brookhaven National Laboratory (BNL). Single dislocations, as well as dislocation nets and highly strained regions were identified in the *topographs*. At least for lysozyme the topographic methods were non-destructive and regular diffraction data collection for structure determination was successfully carried out on the same crystals after the topographic observations.

The temperature and supersaturation regimes during crystal growth, the defect density detected by X-ray topography, and the diffraction resolution obtainable from the crystals were all strongly correlated.

Towards the **third** main objective, we have developed a novel experimental set-up that, for the first time, allowed reproducible *protein crystallization experiments with forced solution flow*. To this end, we have expanded our interferometry setup [Vekilov et al., J. Crystal Growth 146 (1995) 289] by a novel crystallization cell and solution recirculation system. This combination permits monitoring of interface morphology and kinetics with a depth resolution of 200 Å at bulk flow rates of up to 2000 µm/s. Particular attention was paid to the prevention of protein denaturation that is often associated with the pumping of protein solutions. We found that at bulk flow rates $u < 250$ µm/s the average growth rate and step velocity, R_{avg} and v_{avg} , increase with increasing u . This can be quantitatively understood in terms of the enhanced, convective solute supply to the interface. With high purity solutions, u 's > 250 µm/s lead to growth deceleration, and, at low supersaturations σ , to growth cessation. When solutions containing ~ 1 % of other protein impurities were used, growth deceleration occurred at any $u > 0$ and cessation in the low σ experiments was reached at about half the u causing cessation with pure solution. The flow-induced changes in R_{avg} and v_{avg} , including growth cessation, were reversible and reproducible, independent of the direction of the u -changes and solution purity. Hence we attribute the deceleration to the convection-enhanced supply of impurities to the interface, which at higher flow rates overpowers the effects of enhanced interfacial solute concentration. Most importantly, we found that convective transport leads to a significant reduction in kinetics fluctuations, in agreement with our earlier expectations for the lysozyme system [Vekilov et al., Phys. Rev. E 54 (1996) 6650]. This supports our hypothesis that these long-term fluctuations represent an intrinsic response feature of the coupled bulk transport-interfacial kinetics system in the mixed growth control regime.

Refereed Publications:

1. M. Muschol and F. Rosenberger, *Interaction in undersaturated and supersaturated lysozyme solutions. Static and dynamic light scattering results*, J. Chem. Phys. **103** (1995) 10424-10432.
2. B.R. Thomas, P.G. Vekilov and F. Rosenberger, *Heterogeneity determination and purification of commercial hen egg white lysozyme*. Acta Crystallogr. **D52** (1996) 776-784.
3. P.G. Vekilov, L.A. Monaco, B.R. Thomas, V. Stojanoff and F. Rosenberger, *Repartitioning of NaCl and protein impurities in lysozyme crystallization*. Acta Crystallogr. **D52** (1996) 785-798.
4. F. Rosenberger, *Protein crystallization*, J. Crystal Growth **166** (1996) 40-54.
5. F. Rosenberger, P.G. Vekilov, M. Muschol and B.R. Thomas, *Nucleation and crystallization of globular proteins - What do we know and what is missing?* J. Crystal Growth, **167** (1996) 1-27.
6. P.G. Vekilov, J.I.D. Alexander and F. Rosenberger, *Nonlinear response of layer growth dynamics in the mixed kinetics-bulk transport regime*. Phys. Rev. E **54** (1996) 6650-6660.

7. M. Muschol and F. Rosenberger, *Lack of evidence for prenucleation aggregate formation in lysozyme crystal growth solutions*, J. Crystal Growth **167** (1996) 738-747.
8. P.G. Vekilov, H. Lin and F. Rosenberger, *Unsteady crystal growth due to step-bunch cascading*. Phys. Rev. E **55** (1997) 3202-3214.
9. V. Stojanoff, D.P. Siddons, L.A. Monaco, P.G. Vekilov and F. Rosenberger, *X-ray topography of tetragonal lysozyme grown by the temperature controlled technique*. Acta Crystallogr. **D53** (1997) 588-595.
10. F. Rosenberger, P.G. Vekilov, H. Lin and J.I.D. Alexander, *A rationale for system-dependent advantages and disadvantages of solution crystal growth at low gravity*. Microgravity Sci. Tech., **10** (1997) 29-35.
11. M. Muschol and F. Rosenberger, *Liquid-liquid phase separation in supersaturated lysozyme solutions and associated precipitate formation/crystallization*, J.Chem. Phys. **107** (1997) 1953-1962.
12. B.R. Thomas, P.G. Vekilov and F. Rosenberger, *Effects of microheterogeneity on hen egg white lysozyme crystallization*. Acta Crystallogr. **D54** (1998) 226-236.
13. P.G. Vekilov and F. Rosenberger, *Protein crystal growth under forced solution flow: experimental setup and general response of lysozyme*. J. Crystal Growth **186** (1998) 251-261.
14. P.G. Vekilov and F. Rosenberger, *Increased stability in crystal growth kinetics in response to bulk transport enhancement*. Phys. Rev. Lett. **80** (1998) 2654-2656.
15. P.G. Vekilov and F. Rosenberger, *Intrinsic kinetics fluctuations as cause of growth inhomogeneity in protein crystals*. Phys. Rev. E **57** (1998) 6979-6981.
16. F. Rosenberger, *Protein crystallization: specific phenomena and general insights on crystallization kinetics*, Materials Science Forum, **276-277** (1998) 241-256.
17. P.G. Vekilov, B.R. Thomas and F. Rosenberger, *Effects of convective solute and impurity transport on protein crystal growth*. J. Phys. Chem., **102** (1998) 5208-5216.

Invited Presentations

1. P.G. Vekilov, *Impurities- and salt- rich coring in protein crystals*. Seminar, Institute for Materials Research, Tohoku University, Sendai, Japan, November 7, 1995.
2. P.G. Vekilov, *High-resolution interferometry investigations of protein crystals growth*. Seminar, Institute for Materials Research, Tohoku University, Sendai, Japan, November 8, 1995.
3. P.G. Vekilov, *Crystallization under mixed transport and interface control – effects of gravity-driven convection*. Marshal Space Flight Center, Space Science Laboratory, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, January 31, 1996.
4. P.G. Vekilov and F. Rosenberger, *New factors for protein crystal perfection on Earth and under reduced gravity*. Protein Crystal Growth Conference, Panama City, Florida, USA, April 28-30, 1996.
5. P.G. Vekilov, J.I.D. Alexander and F. Rosenberger, *Nonsteady dynamics of layer growth in the mixed kinetics-bulk transport regime*. Tenth American Conference on Crystal Growth, Vail, Colorado, USA, August 3-9, 1996.
6. P.G. Vekilov, *Impurities- and salt- rich coring in protein crystals*. Department of Chemistry, University of Alabama in Huntsville, Colloquium, Huntsville, AL 35899, USA, September 20, 1996.
7. P.G. Vekilov, *System-specific effects of reduced gravity on protein crystal perfection*. Japanese-US Workshop on Protein Crystal Growth in Microgravity, Huntsville, AL, USA, December 16-17, 1996.

8. P.G. Vekilov, *System-dependent advantages and disadvantages of reduced gravity for the quality of protein crystals*. Structural Biology Seminars, Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, AL, May 6, 1997.
9. P.G. Vekilov, *Insight into Transport-Kinetics Coupling Effects in Protein Crystallization from Forced Flow Experiments*. Conference SPACEBOUND '97, Montreal, Canada, May 11-15, 1997.
10. P.G. Vekilov, *Why some protein crystals grow better in space and others don't*. Gordon Conference on Gravitational Effects on Physico-Chemical Systems, Henniker, New Hampshire, USA, June 29-July 4, 1997.
11. P.G. Vekilov, *Nonlinear dynamics of layer growth in the mixed kinetics-bulk-transport regime*. Gordon Conference on Thin Films and Crystal Growth, Plymouth College, New Hampshire, USA, July 5-10, 1997.
12. P.G. Vekilov, *Coupling between transport in solution and interfacial kinetics*. Interdisciplinary Workshop on Phase Transformations Occurring in Solutions of Biological Macromolecules, MIT, Cambridge, MA, USA, October 5-7, 1997.
13. P.G. Vekilov, *Convective supply of solute and impurities in protein crystal growth: microgravity relevance*. Marshal Space Flight Center, Space Science Laboratory, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, January 14, 1998.
14. P.G. Vekilov, *Nonlinear dynamics of layer growth and consequences for protein crystal perfection*. 7-th International Conference on Crystallization of Biological Macromolecules, Granada, Spain, May 3-7, 1998.
15. P.G. Vekilov, *Protein crystal growth - microgravity aspects*. 32nd COSPAR Scientific Assembly, Nagoya, Japan, July 12-19, 1998.

Contributed presentations

1. H. Lin, P.G. Vekilov and F. Rosenberger, *Modeling mass transport and surface kinetics of protein crystals growth*. AIAA 1995 Space Programs and Technologies Conference and Exhibit, Huntsville, AL, USA, September 26-28, 1995.
2. P.G. Vekilov and F. Rosenberger, *Impurities, growth layer sources and kinetics fluctuations in the growth of lysozyme crystals*. VI International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, November 12-17, 1995.
3. P.G. Vekilov, L.A. Monaco, B.R. Thomas, V. Stojanoff and F. Rosenberger, *Impurities- and salt-rich coring in lysozyme crystals*. VI International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, November 12-17, 1995.
4. P.G. Vekilov and F. Rosenberger, *Transport-kinetics coupling effects in protein crystallization studied by forced flow experiments*. American Physical Society March Meeting, Kansas City, MO, USA, March 17-21, 1997.
5. H. Lin, P.G. Vekilov and F. Rosenberger, *Simulation of the interplay between diffusive nutrient transport and nonlinear crystal growth kinetics*. Conference SPACEBOUND '97, Montreal, Canada, May 11-15, 1997.
6. A.R. Feeling-Taylor, P.G. Vekilov, B.R. Thomas, F. Rosenberger, R.E. Hirsch and R. Nagel, *Solubility and crystallization of a mutated hemoglobin, Hb C*, 7-th International Conference on Crystallization of Biological Macromolecules, Granada, Spain, May 3-7, 1998.